BIOPROCESSING OF CRUDE OILS

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INTRODUCTION

Research on using microorganisms to upgrade or improve crude oil properties is a high risk venture but the potential rewards of achieving such a process are significant both environmentally and economically. The main drivers for this work are: 1. tightening of environmental restrictions on total sulfur in refined oil products and lower refinery CO_2 emissions; 2. the diminishing availability of high gravity sweet crudes; 3. the rising cost of coking and hydrotreating operations; and 4. the increasing cost of meeting CO_2 and sulfur restrictions as future emission allowances are reduced.

Coking and hydrotreating operations are refinery processes to upgrade and remove sulfur from heavy crudes. Cokers use heat to remove hydrogen deficient portions of the feed as coke. The process produces a significant amount of carbon waste that must be disposed. Hydrotreating removes feed sulfur and olefins, but requires high pressure equipment, heat, hydrogen, and expensive chemical catalysts. Repeated hydrotreating to achieve very low sulfur levels becomes uneconomic. The main incentives for crude oil upgrading and desulfurization prior to refining are the economics of higher oil value, lower processing costs, and reduced air pollution that causes smog, acid rain and global warming. Refinery processing costs are projected to steadily increase as reserves of high gravity and low sulfur crude feedstocks are depleted because these feedstocks are currently used to dilute heavy oil feeds.

The use of microorganisms or their enzymes provides potential processing advantages of low pressure, low temperature, no hydrogen required, low chemical costs, and minimal equipment investment when compared to refinery operations.

RESULTS AND DISCUSSION

Potentially High Payback

Texaco has significant holdings in heavy and high sulfur crude oil reserves. The value of those reserves can be significantly increased with gravity improvement or viscosity reduction and sulfur removal. An oil field bioprocess that would reduce sulfur would significantly improve the quality and value of oil piped to a refinery. Refining a low sulfur crude would reduce refining costs and achieve lower sulfur product streams. The value of biodesulfurized oil will steadily increase as reserves of low sulfur crude oil are depleted. An estimated $295 million (Table 1) could be realized annually from four selected Texaco's fields if the sulfur content of each oil was lowered to a total sulfur percentage of 0.5% or less. These estimates do not include any savings in refining cost or environmental emission improvements.

Envisioned Advantages of Bioprocessing Crude Oil

Bioprocessing crude oil has several application advantages that make it attractive for use in the oil field prior to shipment or pipelined to a refinery. The first advantage is the potential simplicity of the process. Bioprocessing oil basically involves mixing the water soluble biocatalyst (either the microorganism or the enzyme) with air and the oil. After reaction, the formed water-oil emulsion is separated to recover the upgraded oil. The biocatalyst remains with the water potentially available for reuse. In an oil field, both water and oil are routinely coproduced at the well head as emulsions so separations of oil-water mixtures are standard procedures. The only added feature for bioprocessing is the addition of a mixing reactor prior to separation. Fit of such a bioreactor in an oil field operation are illustrated and described below.

A second advantage of bioprocessing is the selectivity of biocatalysts. Desulfurizing biocatalysts contain enzymes generated by microorganisms that need sulfur for energy and growth. Specifically, these generated enzymes catalyze oxidative cleaving of sulfur atoms from organic sulfides forming sulfate salts. The sulfate salts then migrate from the organic oil phase to the water phase. These microorganisms can be modified to remove specific sulfur structures or broader classes of sulfur compounds. Biocatalysts that can upgrade oils modify specific organic structures like cleaving or opening aromatic rings. The motivation for the organism again is to obtain chemical components for energy and growth. The advantage of this selectivity is that a process user knows what chemical changes or potential losses will occur. Unfortunately, crude

oil is a complex mixture of organic compounds and all crude oils do not have the same chemical makeup. Therefore, for crude oil applications the biocatalysts will have to be customized for each crude. The total process may involve multiple biocatalysts. Again, the oil field application is felt to be more suited to bioprocessing than at a refinery where multiple crude feeds are handled.

A third advantage is that the bioprocessed oil can be processed at a refinery using current technology at a lower cost. For sulfur, hydrotreating is very effective for removing mercaptans and straight chain sulfides, whereas biodesulfurization is more effective with organic ring sulfides like dibenzothiophene (DBT). The hydrotreating conditions are less severe (lower cost) for straight chain sulfur compounds so an economic advantage is achieved when both hydrotreating and biodesulfurization processes are used.

A fourth advantage of a bioprocessing is improvement in oil properties that affect handling the bulk oil. These property improvements could include viscosity reduction, shifts to lower molecular weight distributions, or lower asphaltene content all of, which reduce fluid piping and transportation problems and costs. Basically, a higher grade and cleaner oil (low sulfur content and high gravity) is transported and refined cheaper and lowers sulfur and CO_2 emissions.

Process Location: Refinery *vs.* Field

The bioprocess can be applied either on a crude oil in the field or later at the refinery. Five factors will be considered in comparing the advantages and disadvantages of applying biodesulfurization near the well head location or at a refinery location. The five comparison factors are greatest product value, biocatalyst solubility, reaction time, process integration, and waste stream disposal. These five points are basic, but they illustrate the envisioned application of bioprocessing a crude oil.

Processing costs will have the greatest effect on product values where the pricing margins are the smallest. In other words, one is more inclined to pay the cost of processing where the greatest product value increase can be obtained. For example, the value of a desulfurized crude oil could increase from $1 - $3/barrel assuming sulfur is reduced to less than 0.5%. In a refinery, product profit margins are squeezed by competition and environmental regulation costs. In general, refined product margins are usually much lower than $1.00/barrel. The maximum benefit of desulfurizing occurs closest to the well head based on improvement in product value.

The current envisioned biocatalysts for crude oil upgrading and desulfurization are water soluble. In an oil field operation, water is coproduced with the oil so the process of water/oil separation is a routine field process. The agitation of the oil and water mixture with biocatalyst would be the only added process step. However, prior to oil transportation to a refinery most of the water has been removed to minimize corrosion and viscosity problems. Water/oil mixing at a refinery is minimized to prevent water and emulsion carry over into the oil processing. Water/oil mixing followed by separation would be added process steps which means added capitalization and operating costs. Again, oil bioupgrading in the oil field has advantages over refinery processing.

Longer reaction times or longer biocatalyst contact with the oil results in greater oil property improvement and sulfur removal. In an oil field setting, the reaction time can range from hours to days depending on oil production rate, storage capacity, and shipment timing. Bioprocessing reaction times allowable in a refinery operation are limited to hours or less. Material holding times at a refinery are limited and most refineries maintain continuous operation and high throughput unlike a field operation where in some cases oil can be held in a tank for several days awaiting transportation.

Another major difference in chemical processing between a refinery and a field situation is the quantities of material handled. A refinery deals in volumes greater than 50,000 gallons whereas a producing field generally works with volumes less than 50,000 gallons. The size aspect with addition of process steps affects the ease of integration of bioprocessing to a refinery or a field facility. For the field case, the process integration is relatively simple. The field already has holding tank capacity and necessary oil/water separators. Added equipment is a mixing reactor. Process integration for a refinery is similar but on a much larger scale with accompanying higher cost.

A bioprocessing operation will generate a waste water stream. All sulfur removed is converted from an organic form to a sulfate salt which is soluble in the reaction water. The biocatalyst will be removed from the water and recycled but the remaining process water will contain metal, sulfur, and organic salts. The new water waste stream must be handled whether it is generated in the oil field or at the refinery. In a refinery, this new waste stream becomes an added problem.

However, in the field the water containing the formed salts can be diluted and reinjected as part of the field water flood program so waste stream has minimal effect on oil field operation. The water soluble salts may also have minimal surfactant properties which could improve the water flood sweep efficiency.

Generic Oil Field Production Facilities

A basic field production facility is illustrated in Figure 1. As the oil and water are produced accompanying gas is separated and collected for sale. The water oil mixture or emulsion is then sent to a heater treater also called a gun barrel to separate the oil and water. Any residual gas released during separation is used to blanket the rest of the separation system. The separated water and oil are transferred to separate tanks. The oil is collected for sale and transportation and the water is filtered and reinjected as part of the field water flood. A bioprocessing step can be inserted after the initial gas separation. The biocatalyst can be added to the produced water/oil emulsion and mixed. After a sufficient reaction time the oil/water emulsion would be handled as before in the field separating facility as illustrated.

Bioprocessing Results

Biodesulfurization results on a crude oil are illustrated in Figure 2. The curves are GC detector signals from a sulfur specific detector (Sievers Model 355) where the Y-axis is the detector signal and the X-axis is time in minutes. The lower curve with the single peak labeled DBT is a dibenzothiophene standard. The next two curves are the biodesulfurized oil and original oil respectively. The reaction period was 24 hours at room temperature. A reduction in sulfur content is indicated.

CONCLUSION

The potential benefits and payback justify further research on using microorganisms to upgrade crude oil. There are distinct advantages in applying a crude oil bioupgrading process in the oil producing fields rather than a refinery. Lower process temperatures, lower reaction pressures, and more environmentally friendly waste streams are a significant paradigm shift from current petroleum technology.

TABLE 1 - Estimates of the Increased $/Yr. by Reducing Sulfur in Crude Oil

A	$900,000
B	$29,200,000
C	$120,450,000
D	$146,000,000
Total	$296,550,000

Estimates based on $1 - $3 per bbl increase in price for low sulfur crude

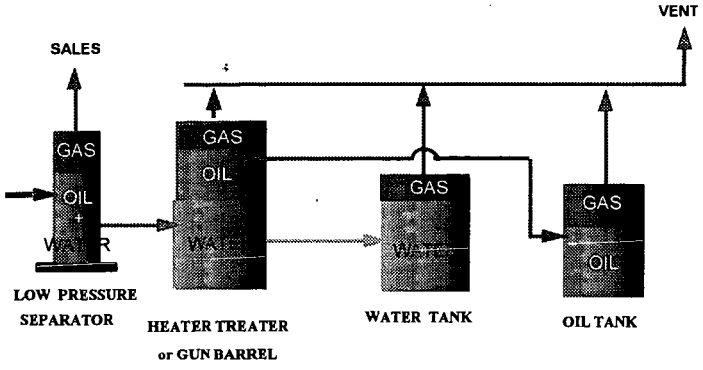

FIGURE 1 - Oil Production Facility

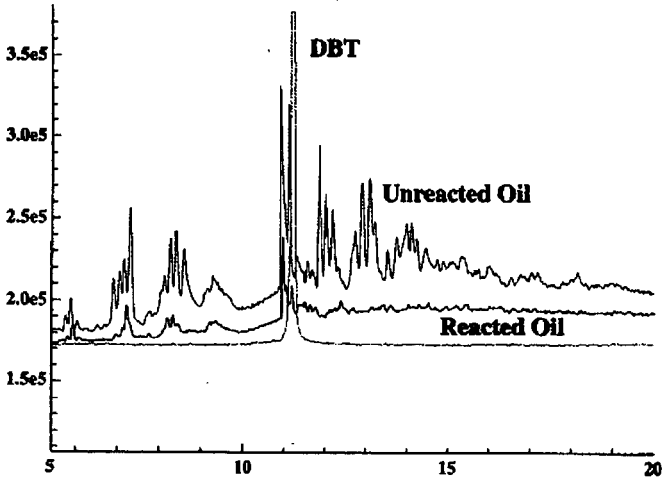

FIGURE 2 - Biodesulfurized Crude Oil Result

BIOCATALYTIC CONVERSION OF CRUDES: POSSIBLE PATHWAYS

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INTRODUCTION

Petroleum is a complex mixture of natural products which, under geological conditions (temperature, pressure, folding, migration and others), are subjected to multiple chemical, biochemical and physical reactions (1). Evolution of petroleum over geological periods of time leads to the formation of mixtures of hydrocarbons with different molecular weights and concentrations of saturates, aromatics, resins, asphaltenes and organometallics. Thus, low °API (<20) gravity oils are low in gasoline and high in residuum, i.e., as oils becomes heavier, the H/C ratios decrease, the NSO/C ratios increase and the oil becomes richer in asphaltenes and (2) Treatment of such complex mixtures with biocatalysts introduced under controlled conditions leads to bioconversion of crude oils via multiple inter- and intra-molecular chemical and biochemical processes. Some of the mechanistic aspects of such processes will be discussed.

Chemical reactions leading to the formation of different types of oils are influenced by the composition of the initial mix of natural products, their mutual interaction involving multiple reactive sites and the effects of chemical, biochemical and geological factors acting on the mixture (2,3). In spite of the chemical complexity of crude oils they can all be characterized and placed into distinct groups ranging from lights oils (°API >30) to heavy oils (°API <20). They are further distinguished by a number of chemical markers, compounds characteristic of major components of crudes which are different hydrocarbons, grouped according to their particular chemical structures: saturates, aromatics, resins and asphaltenes. Additional chemical characterization of oils distinguishes complex hydrocarbon mixtures by the relative concentration of compounds containing nitrogen, sulfur, oxygen and trace metals. The representative uniqueness of chemical markers has allowed to follow the chemical alterations in crude oils under reservoir as well as chemical and biochemical processing conditions (4-7). The latter is of particular importance in the studies dealing with biocatalytic conversion of heavy crude oils (e.g., Ref. 7). It has been shown by extensive use of chemical markers, that biochemical catalysts do not biodegrade, but rather, bioconvert heavy crudes to products with lower contents of sulfur, nitrogen and trace metals (7,8). Further, the extent and the specificity of bioconversion depends on the biocatalyst used and the chemical properties of the crude used, for example the difference between a heavy immature and a heavy biodegraded crude oil. Some aspects of the above observations will be briefly discussed in the present paper.

EXPERIMENTAL

Methods and procedures have been discussed in detail elsewhere (e.g, 7,9) and will be summarized in this section. .1. Biocatalyst and oil treatment: biocatalysts were prepared from the stock BNL collection and stirred into the reaction mixture containing the oil and nutrients and then extracted with methylene chloride; .2. gas chromatography/mass-spectrometry: the analyses were carried out with a Perkin Elmer Model 8700 gas chromatograph equipped with flame photometric detector (FPD) and a Finnigan ion trap mass spectrometer (ITD) for simultaneous analyses (GC-FPD/MS); .3. pyrolysis-gas chromatography-mass spectrometer (Py-GC-MS) utilized a Chemical Data System, Inc. (CDC) model 190 pyroprobe interfaced with GC-FPD-MS system as in .2.; .4. for nitrogen analyses appropriate nitrogen specific (nitrogen, phosphorous detector, PFPD) was used; .5. metals analyses were performed with a VG-induced-coupled-mass spectrometer (ICP-MS); .6. Saturate Aromatic, Resins and Asphaltene (SARA) analyses were carried out using a thin layer Chromatograph-Flame Ionization Detector (TLC-FID) and Chromatotron Model TH-10 of IATROSCANCO, using the rotating disc TLC-FID method (10) and .7. asphaltenes were precipitated with n-pentane from a methylene chloride solution.

RESULTS AND DISCUSSION

Experimental results to date suggest that a biocatalyst reacts at the active sites of polar molecules within the colloidal, micellar molecular solutions representative of three dimensional

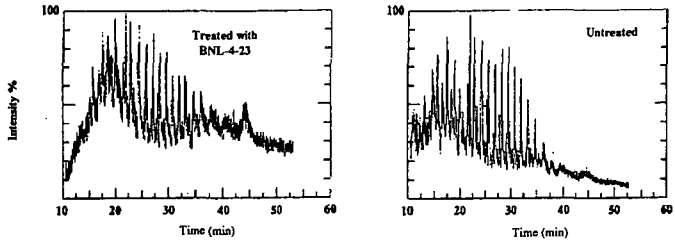
structures of crudes (7-14). It is the three dimensional structure of crudes as well as the chemical composition and the molecular structures of major constituents of oil that influence the reactivities of crude oils (15, 16). It has been known for some time (14) that measurement of one gross parameter (e.g., sulfur) in analyses of catalytically converted oils, for example hydrodesulfurization and hydrodemetalization is insufficient, and may lead to erroneous conclusions. Current experience suggests that similar oil characterization scenarios apply to biocatalytically converted oils. For example, the action of a single biocatalyst, BNL-4-23 on three, chemically different oils is summarized in Table 1. Monterey 851 is a heavy, California (onshore) biodegraded oil. OSC is an immature (offshore) biodegraded oil, while MWS, Midway Sunset is a steam treated heavy crude. There is a significant variation in the effects of a single biocatalyst on eight different parameters. Similar variations have been also observed where several different biocatalysts have been used on a single oil (examples in Refs. 6,7 and others). Further, the distribution of compounds containing active sites, i.e., heteroatoms, varies in concentration throughout different types of oils, where the three dimensional structure of oils allows for formation of bridges, clathrates, inclusion complexes and clusters which, chemically represent a reaction substrate eminently suitable for multiple and simultaneous reactions. The importance of initial reactivity at active sites has led to a set of experiments described in detail elsewhere (6-9) and will be for the purpose of this discussion, summarized briefly in Figure 1. In the analyses shown in Figure 1, experimental conditions for all the samples were kept constant in each case, so that a direct comparison of spectral results is possible. These results show that significant changes also occur in the n-pentane precipitated fractions (asphaltanes) which are not detected in the analyses of the whole oil. The chemical changes which lead to reaction mixtures analyzing as shown in Figures 1c, d, g, h indicate a break down in high molecular weight fractions. The multiple parameter analysis shows lowering in total sulfur concentration and also indicates a significant re-distribution of hydrocarbons.

The usefullness and importance of multiple parameter analysis as it relates to an understanding of fundamental mechanisms by which biocatalysis occurs as well as to its effects and efficiency is further illustrated by the following examples. Table 2 lists data for several fractions of different oils with their corresponding Conradson Carbon Residue (CCR) values. When CCR values are plotted against % of resins + asphaltenes, a decrease in the concentration of resins and asphaltenes also indicates a decrease in the Conradson Carbon value, an important consideration in refining of crudes, i.e., coking processes. The effect of biocatalytic conversion of OSC and MWS follows the same trend, however, biocatalysis accomplishes this at a much lower temperature and pressure compared to conventional refining. This very important experimental observation could not have been detected without multiple parameter analyses and systematic studies of chemical/biochemical mechanisms associated with biocatalysis.

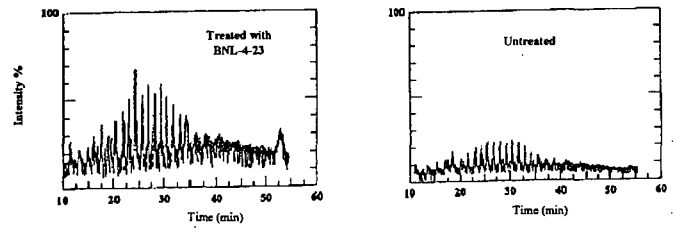
Table 1.
Characterization of Representative Biotreated Crude Oils

Oil	Monterey 851		OSC		MWS	
	%UN*	% TR** BNL-23	%UN	% TR BNL-23	%UN	% TR BNL-23
S	1.84	1.29 (-30)	4.4	2.42 (-45)	1.00	0.50 (-50)
N	0.59	0.44 (-25)	0.66	0.36 (-45)	0.79	0.63 (-20)
Ni	259 ppm	207 (-20)	80 ppm	64 (-20)	63 ppm	47 ppm (-25)
V	369 ppm	288 (-22)	22 ppm	18.5 (-16)	24 ppm	15.9 ppm (-36)
SAT %	19.2	34.4	17.3	51.6	19.2	66.3
AROM %	45.2	29.7	39.1	20.5	44.9	11.2
RESIN %	31.2	32.7	37.1	22.3	35.3	19.3
ASPH %	4.4	3.6	6.2	5.7	2.60	3.1

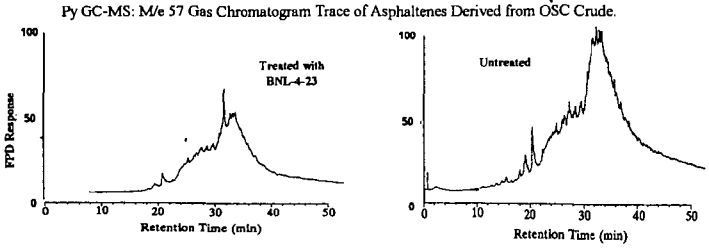
* UN = untreated
**TR = treated
(-) = % reduction



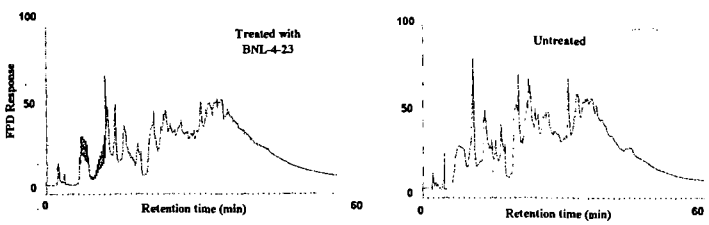
GC-MS Analysis: M/e 57 Gas Chromatogram Trace of Untreated and Treated OSC Crude.



Py GC-MS: M/e 57 Gas Chromatogram Trace of Asphaltenes Derived from OSC Crude.



FPD (Sulfur Specific Detector) Chromatogram Trace of Untreated and Treated OSC Crude.



Pyrolysis-gas Chromatograph Analysis (Py-GC) FPD (Sulfur Specific Detector) Trace of Asphaltenes from OSC Crude Treated with BNL-4-23

Figure 1. *Multiple Parameter Analysis of Oils.*

Table 2.
Distribution of Fractions and Residues in Representative Oils

Reference		Asphaltenes	Resins	Res+Asph.	Carbon residue
2	Light Alberta	0.2	9.4	9.6	1.3
2	crudes	0.1	11.6	11.7	2.6
2	for comparison	0.9	12.2	13.1	2.9
2		0.3	13.0	13.3	3.0
17	Daquig	0	29.9	29.9	8.5
15	Gach Saran	6.8	28.5	35.3	8.8
2	Boscan	11.9	24.1	36.0	10.4
15	Heavy Arabian	12.6	27.5	40.1	12.6
2	Cold Lake	15.7	28.7	44.4	13.6
17	Shenghi	45.7	1.6	47.3	12.7
15	Blend	11.4	36.0	47.4	11.0
2	Bermudez Lake	35.3	14.4	49.7	13.4
2	Athabasca	16.9	34.1	51.0	18.5
15	Maya	25.2	25.9	51.1	15.3
2	Lloydminister	12.9	38.4	51.3	11.8
17	Renqiu	0	53.1	53.1	18.6
15	Hondo	13.9	40.2	54.1	10.8
2	Qayarah	20.4	36.1	56.5	15.6
2	Trinidad Lake	33.3	29.4	62.7	10.8

2-Speight
14-Dolbear, Tang and Moorhead
16-Chan and Luoyan

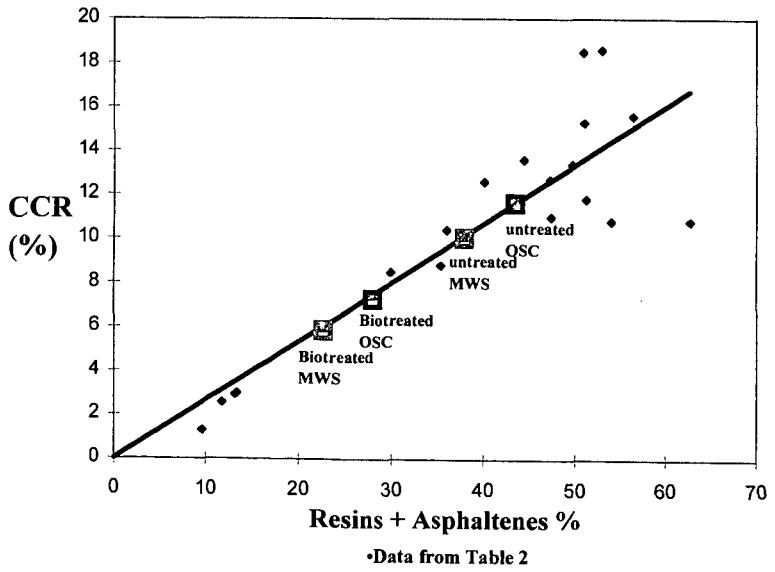


Figure 2. Conradson Carbon Residue (CCR) as a function of the Resin + Asphaltene Fraction

CONCLUSIONS

Multiple chemical changes which occur during biocatalysis of heavy crude oils are due to complex inter- and intra-molecular reactions. These reactions follow distinct trends which can be followed by a set of chemical markers associated with major heavy crude oil fractions. Experience gained with chemical catalytic conversion of crudes, shows that properties described by a single paramter only (e.g., sulfur) are not in themselves good predictors of catalytic processing. The data currently available for biocatalytic conversion of heavy crudes appear to be consistent with those of chemical catalysis and require an array of chemical markers in evaluation of biocatalytic effects.

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BIOCATALYTIC RING CLEAVAGE OF DIBENZOTHIOPHENE AND PHENANTHRENE IN A BIPHASIC FERMENTER SYSTEM

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Keywords: Biotransformation, petroleum, fermenter

ABSTRACT

Our previous research has demonstrated the ability of *Pseudomonas fluorescens* LP6a to cleave the fused-ring aromatic hydrocarbons and heterocycles commonly present in petroleum distillates. In this study, experimental conditions for biocatalytic ring cleavage of dibenzothiophene and phenanthrene in a biphasic 3 liter fermenter system were investigated.

Compared with batch flask experiments, a fermenter system provides the advantage of large scale, easy automation and versatile control of the experimental conditions. Growth curves of the biocatalyst and the relative oxidation rate of dibenzothiophene at different growth phases were determined. Incubation of dibenzothiophene and phenanthrene in various carriers was performed in the fermenter system. Rapid complete biotransformation of dibenzothiophene in light mineral oil and improved biotransformation of phenanthrene in diesel fuel were obtained in a fermenter system compared with batch flask conditions.

INTRODUCTION

Petroleum middle distillates contain low fuel value di- and tricyclic aromatics. Currently, expensive and non-specific chemical hydrogenation processes involving high temperature and high pressure are used to reduce di- and tricyclic aromatics to alkylbenzenes to increase their fuel value. It is desirable therefore to explore other economical alternatives to conventional upgrading techniques.

Our previous research has demonstrated the ability of *Pseudomonas fluorescens* LP6a to cleave fused-ring aromatic hydrocarbons and heterocycles commonly present in petroleum distillates [Wu et al., 1998]. This provides an attractive potential alternative to conventional fuel upgrading techniques as a two-stage upgrading process: first bacteria would enzymatically cleave the fused-ring aromatics under near-ambient conditions, then mild chemical hydrogenation of the ring opening products would yield the desired alkyl aromatics.

We previously demonstrated ring cleavage of aromatics in middle distillates under batch flask conditions, typically using 200 mL of biocatalyst suspension and <1 mL of distillate [Foght et al., 1997; Wu et al. 1998]. To scale up the reaction volumes, experimental conditions for biocatalytic ring cleavage of dibenzothiophene and phenanthrene in a 3 L fermenter system were investigated in this study. A biphasic system was used, with the ring cleavage substrate dissolved in a water-immiscible carrier and the biocatalyst suspended in an aqueous buffer.

Compared with batch flask experiments, a fermenter system provides the advantage of large scale, automation and versatile control of the experimental conditions such as aeration and mixing. Growth curves of the biocatalyst and the specific rate of dibenzothiophene (DBT) ring cleavage at different growth phases were determined. Results are presented from biotransformation of the model ring cleavage substrates dibenzothiophene or phenanthrene dissolved in light mineral oil, diesel fuel or a petroleum middle.

METHODS AND MATERIALS

Preparation of biocatalyst

A transposon mutant of *P. fluorescens* LP6a was generated by conjugation with a suitable *Tn*5 donor plasmid [Foght and Westlake, 1996] and subsequent screening for desired ring cleavage products. Cultures of *P. fluorescens* LP6a mutant #21-41 were incubated to high density at 30°C with agitation for 24 h in Tryptic Soy Broth (Difco; typically 200 mL) containing kanamycin to maintain the transposon. The 200 mL seed culture was then used to inoculate a fermenter (New Brunswick, NJ) containing 3 L Tryptic Soy Broth plus kanamycin. The culture was incubated at 30° C with aeration at 4 L min^{-1} sterile air and stirring at 300 rpm.

To determine optimum biocatalyst preparation conditions, the culture optical density at 600 nm and protein concentration (BCA protein assay; Pierce, Rockford IL) were determined at intervals in the fermenter culture. As well, approximately 50 mL subsamples of the growing culture were assayed for ring cleavage activity, using crystalline dibenzothiophene (DBT) as the substrate. The

rate of DBT biotransformation was determined spectrophotometrically at 475 nm, the wavelength of maximum absorbance of the DBT ring cleavage product [Kodama et al., 1973]. Thereafter, for convenience, the fermenter cultures were grown to stationary phase overnight (ca. 18 h) under the same conditions.

Enzymatic ring cleavage activity was induced in the grown culture by adding salicylic acid [Yen and Serdar, 1988]. The induced cells were harvested with a high speed continuous centrifugation system (CEPA; New Brunswick, NJ) and resuspended in potassium phosphate buffer (pH 7) to the same density as the original grown culture. This suspension was used in the same fermenter system as the "active biocatalyst", and subsequently incubated with a model substrate dissolved in a carrier.

Model substrates and carriers

Quantitative analysis of specific changes in the composition of petroleum products is difficult due to the chemcial complexity of the substrate. Therefore, we chose to "spike" petroleum products with individual model substrates and quantify their ring cleavage against the complex background. Phenanthrene and DBT were selected as model substrates for biocatalysis, representing tricyclic and heterocyclic aromatic substrates, respectively. DBT was dissolved in 20 mL of light mineral oil while phenanthrene was dissolved in 30 mL of authentic hydrotreated middle distillate HP16 and straight run diesel and added to the suspension of induced biocatalyst. This reaction mixture was incubated with aeration and agitation for 24 h to effect biotransformation of the substrates. Parallel cell-free controls were also prepared and compared with the biocatalytic samples.

Analytical methods

The rate of biotransformation of DBT dissolved in light mineral oil was determined spectrophotometrically at intervals during biocatalysis by clarifying ca. 1 mL of reaction mixture and measuring absorption at 475 nm. Additionally, 10.0 mL samples were removed and the internal standard was added for pentane extraction of residual DBT and quantitative gas chromatography (GC) [Wu et al., 1998]. The percentage of DBT biotransformed was then calculated by difference.

Similar extraction procedures were used during biocatalysis to recover residual phenanthrene dissolved in HP16 or straight run diesel. Quantitative GC was used to estimate the percentage of phenanthrene biotransformed, using biphenyl and benzothiophene as internal standards to determine the relative residual mass of the model substrate in the pentane extracts. The biotransformation percentage was determined by comparing the relative residual mass of the model substrate recovered after incubation with the biocatalyst to that of the model compound in a parallel sterile (cell-free) control.

Routine GC analysis of fractions and distillates was performed on a Hewlett-Packard model 5890 GC system equipped with a flame ionization detector (FID) and a sulfur-selective flame photometric ionization detector (FPD). Chromatography conditions have been described previously [Foght and Westlake, 1996].

RESULTS AND DISCUSSION

Specific ring cleavage activity of the biocatalyst during culture growth

It was necessary to determine the point in the growth curve at which a growing culture should be harvested for use as a biocatalyst. We observed a short culture lag time in the inoculated fermenter, with stationary phase achieved after 6 to 8 h incubation (Figure 1). The absolute DBT ring cleavage rate of this growing culture increased during incubation (data not shown), but the specific DBT oxidation rate (i.e. the ring cleavage rate normalized to biomass protein) achieved a maximum after 6 hours (Figure 1), i.e. in stationary phase. For convenience, we chose thereafter to standardize culture growth to an overnight incubation (ca. 18 h).

Biotransformation of DBT dissolved in light mineral oil by pre-grown, induced biocatalyst

Light mineral oil was chosen as the carrier for this experiment because it approximates the aliphatic fraction of petroleum products and does not interfere with quantitative GC analysis of the residual DBT.

A rapid decrease in the residual DBT was observed in the biphasic fermenter system. By 7 hours incubation with the biocatalyst, less than 10% of the DBT remained, and within 20 hours the DBT was completely removed (Figure 2). Concomitant accumulation of the DBT ring cleavage

product, monitored spectrophotometrically at 475 nm, reached a maximum at ca. 8 h. then decreased, most likely due to side-reactions of the ring cleavage product.

Biotransformation of phenanthrene dissolved in middle distillate HP16 or straight run diesel by pre-grown, induced biocatalyst

Biotransformation of the model substrate phenanthrene incubated with biocatalyst in a biphasic fermenter system is shown in Figure 3. Middle distillate HP16 or diesel were used as carriers. Unlike DBT biocatalysis (Figure 2), complete removal of phenanthrene was not achieved within 22 h. However, significant biotransformation was observed, with similar initial rates for both carriers. The middle distillate permitted slightly better biotransformation than the diesel (ca. 80% versus ca. 60% substrate removal). The fermenter system achieved better biotransformation than typical batch flasks, which achieved 60% removal of phenanthrene dissolved in middle distillate HP16 in 24 h [Wu et al., 1998].

CONCLUSIONS

Preparation and use of the biocatalyst in fermenter system is rapid and easily controlled using aeration, agitation and temperature. In a single fermenter the biocatalyst can be grown, induced, then re-suspended in buffer (after harvesting) for incubation with the substrate. Biotransformation of model substrates in various carriers was achieved at rates surpassing those achieved in small volume batch flasks, possibly due to superior mixing in the fermenter. The biphasic fermenter system is promising and warrants further research for optimization of operating conditions and for scaling up to larger volumes.

ACKNOWLEDGMENTS

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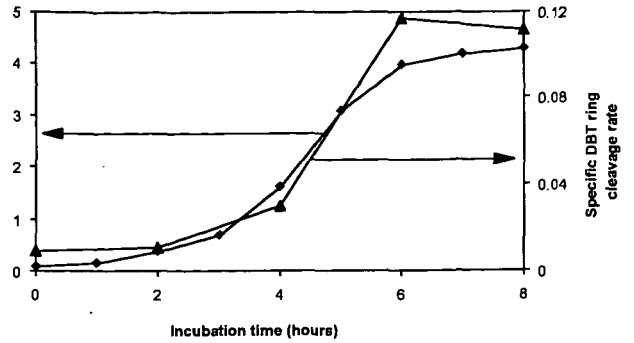

Figure 1. Correlation of culture growth and DBT ring cleavage activity. Growth curve of *P. fluorescens* #21-41 at 30°C measured as optical density at 600 nm (OD_{600}), and specific DBT ring cleavage rate (absorption units at 475nm•min^{-1}•mg biomass $protein^{-1}$) during growth.

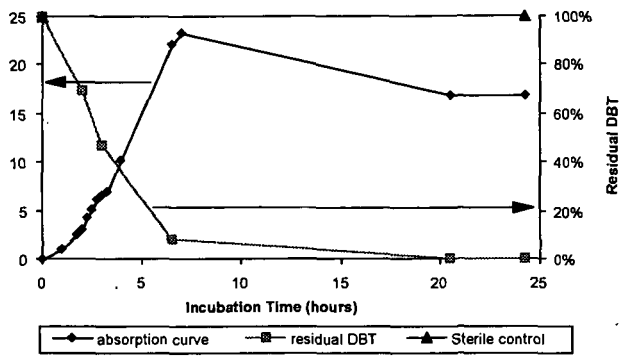

Figure 2. Biocatalysis of DBT and formation of ring cleavage product. Biotransformation of DBT dissolved in light mineral oil by *P. fluorescens* LP6a #21-41 and accumulation of its ring cleavage product, measured at 475 nm; residual DBT measured quantitatively by GC.

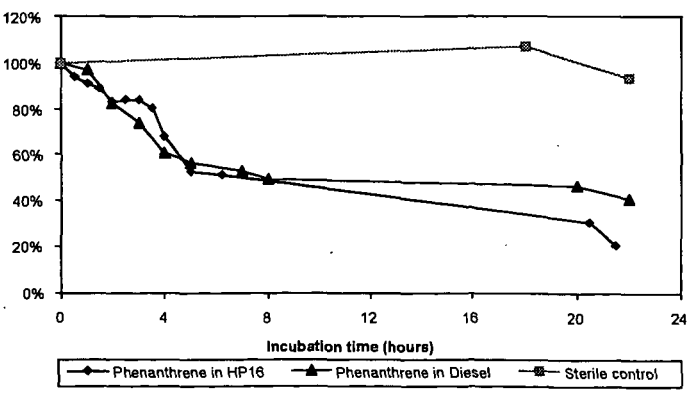

Figure 3. Biocatalysis of phenanthrene in biphasic fermenter system. Biotransformation of phenanthrene dissolved in middle distillate HP16 or diesel fuel by pre-grown, induced biocatalyst, measured by quantitative GC as removal of residual phenanthrene.

MICROBIAL DENITROGENATION OF FOSSIL FUELS

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ABSTRACT

The microbial degradation of nitrogen compounds from fossil fuels is important because of the contribution these contaminants make to the formation of nitrogen oxides (NOx) and hence to air pollution and acid rain. They also contribute to catalyst poisoning during the refining of crude oil, thus reducing process yields. In this study carbazole was used as the model nitrogen aromatic and 1-methylnathphalene was used as the model fuel. Two-phase bioconversions were carried out with carbazole-degrading strains to investigate the feasibility of microbial upgrading of fossil fuels by the removal of nitrogen aromatic contaminants.

INTRODUCTION

Several heterocyclic aromatic nitrogen compounds are found in fossil fuels[1]. Crude oil is a heterogeneous mixture of organic molecules including all-hydrocarbon alkanes and aromatics, as well as sulfur-, oxygen-, and nitrogen-containing heteroaromatic compounds. Many applications of crude oil are hindered by the presence of sulfur-, oxygen-, and nitrogen-containing aromatic compounds. The nitrogenous compounds found in crude oils fall into two classes. The 'nonbasic' molecules include pyrroles and indoles but are predominantly mixed alkyl derivatives of carbazole, while the 'basic' molecules are largely derivatives of pyridine and quinoline (Fig. 1). The total nitrogen content of crude oils averages around 0.3% of which the nonbasic compounds comprise approximately 70-75% (Petrolite). As existing supplies of high quality, low-boiling-point crude decrease, there is a trend towards the use of lower-volatility oils with higher nitrogen contents.

The removal of aromatic nitrogen contaminants from petroleum is important for many reasons. First, their combustion leads directly to the formation of nitrogen oxides (NOx); emissions of NOx, which contributes to acid rain, are under increasingly stringent control by environmental regulations[2,3]. Second, the presence of aromatic nitrogen compounds can lead to significant poisoning of refining catalysts, resulting in a decrease in yield. Carbazole, the major nonbasic species, directly impacts the refining process in two ways: (1) it is converted during the cracking process into basic derivatives that can adsorb to the active sites of the cracking catalyst; and (2) it is unexpectedly potent as a direct inhibitor of hydrodesulfurization, which is commonly included in the refining process in order to meet sulfur content criteria[4-6]. The practical consequence of this catalyst poisoning is that the removal of carbazole and other nitrogen species can significantly increase the extent of catalytic-cracking conversion and the yield of gasoline. With a 90% reduction in nitrogen content, an increase in gasoline yields of up to 20% may be achievable (Petrolite), which would represent a major economic improvement in low-margin, high volume refining processes. Finally, the presence of nitrogen compounds promotes the corrosion of refining equipment such as storage tanks and piping, which adds to the refining costs[7].

Nitrogen heteroaromatics can be eliminated from petroleum using high pressure, high temperature hydrotreating, but such processes are expensive and hazardous, and also modify many other constituents of petroleum. Current research on microbial denitrogenation focuses on the degradation of the nonbasic nitrogen compounds and their alkyl derivatives, because they represent the majority of the total nitrogen and the basic nitrogen compounds can be readily extracted if desired. Although solvent extraction methods also exist for the nonbasic species, approximately 30% of the oil is retained in the extract phase. Such solvent treatments are thus ill-suited to the efficient removal of the nitrogen content.

We believe that microbial transformation of nitrogen heteroaromatics can be used to alleviate catalyst inhibition in several ways. Carbazole, for example, can be completely metabolized to CO_2 and biomass, or (using appropriate blocked mutant strains) converted to anthranilic acid or other intermediates. These appear likely to cause less catalyst inhibition than their parent compound, and many polar intermediates could be readily extracted from petroleum streams into water. It has been reported that carbazole enrichment cultures are capable of degrading a wide range of alkylcarbazoles present in crude oil, generally yielding water-soluble, nontoxic metabolites[8].

In this study Pseudomonad LD2 received from Prof. Phil Fedorak's at the University of Alberta was used due to its extensive metabolic characterization[9] and its high activity relative to other isolates obtained in our lab. Based on published reports of cloning of carbazole degradaing activity in the literature[10,11], the carbazole genes were cloned and mobilized to several other strains of bacteria for two-phase experiments. Many pseudomonads were selected for known solvent resistance[12]. In other model systems based on dibenzothiophene, hexadecane was used as the model fuel. However, given the low solubility of carbazole in hexadecane (~0.03 wt%), 1-methylnapthalene proved to be a better choice due to higher solubility (~0.8 wt%), low freezing point (-22°C), and ease of emulsion separation in the two-phase system. While 1-methylnapthalene proved to be a superior solvent, the toxicity of the oil phase to the microorganisms was problematic compared to hexadecane. Thus several organisms were tested in the model system to find a strain compatible with the 1-methylnapthalene two-phase system.

MATERIALS AND METHODS

Microorganisms and media

A pure culture of Pseudomonas sp. LD2 was obtained from Phil Fedorak's lab at the University of Alberta. Other Pseudomonas strains were obtained from Dr. Jurtshuk at the University of Houston. The growth medium for the culturing of the microorganisms had the following composition (g/L) Tryptone 10, Yeast Extract 10, K_2HPO_4 5, and glycerol 10. Trace metals solution was added at 5 ml to 1 L and had the following composition (g/L) $MgSO_4$ 4.0, NaCl 0.2, $FeSO_4*7H_2O$ 0.2, $MnSO_4*4H_2O$ 0.2, D.I. H_2O 100ml. Solid media were prepared by adding 15 g/L agar to LB medium. LB medium had the following composition (g/L) Tryptone 10, Yeast Extract 5, NaCl 10.

Growth conditions

Seed cultures were started by inoculating 5 ml of media with a sterile loop dipped in -80°C frozen seed cultures with 25% glycerol. The seed cultures were incubated at 30°C in a water bath shaker for 6-8 hours or until approximately 1-2 OD was reached. One ml of this seed culture was added to 1L of media in a 2.8L Fernbach baffled shake flask and incubated at 30°C and 250rpm. After 12-16 hours of growth, the cells were harvested by centrifugation at 4,000 rpm in a Beckman J6B. The supernatant was discarded, and the cells were resuspended in 500 ml of LB for use in two-phase experiments.

Two-phase model system

Thirty ml of cell slurry was added to a 300 ml baffled shake flask. To this flask, 10 ml of 1-methylnapthalene with 0.8 wt% carbazole was added. In hexadecane experiments, 10 ml of carbazole-saturated hexadecane were added. These flasks were incubated at 30 °C and 250 rpm. Time samples were taken by removing a flask and emptying the contents into a 40 ml polypropylene centrifuge tube. The sample was then centrifuged at 15,000 rpm for 30 minutes. After centrifugation a disposable pipette was used to remove approximately 2 ml of the 1-methylnapthalene layer. The 1-methylnapthalene phase was added to an amber 1.5 ml GC sample vial for analysis.

GC analysis

The 1-methylnapthalene phase was analyzed on a HP 6890 gas chromatograph. One µl of sample was injected on a HP-5 column at an initial column temperature of 160°C. The method held the column at the initial temperature for 2 minutes then increased the temperature at a rate of 8°C/min until 250°C was attained. This final temperature was held for 2 minutes. The detector was a NPD, which is specific for nitrogen.

Results and Discussion

Several strains were tested, and the results are summarized in Table 1. All strains tested in the hexadecane model systems showed removal of a low level (~0.03 wt%) of soluble carbazole. However, only one strain showed any ability to remove carbazole in the 1-methylnapthalene two-phase system. None of the tested strains were observed to grow on 1-methylnapthalene as the sole source of carbon. Thus, the 1-methylnapthalene did not supply a co-metabolic substrate for carbazole degradation. In the systems containing *P. fraggi*, the total observed removal of carbazole was 4.7% after 3 hours, 11.3% after 18 hours, and 13.3% after 28 hours relative to the stock solution. An additional flask at 45 hours did not show any further decrease in carbazole concentration relative to the stock solution. In the hexadecane experiments, no carbazole was detected after 2 hours of incubation. No intermediates of carbazole metabolism were detected in

the GC analysis in either of the model system runs. However, control samples analyzed by GC were able to detect anthranilic acid, which is a known intermediate in carbazole degradation[13]. The control flask not containing microorganism did not show any change in carbazole concentration relative to the stock solution. A specific activity was not calculated since growth occurred during the reaction, and the 1-methylnapthalene prevented measurement of optical density or dry cell weight.

CONCLUSIONS

The investigation of biodenitrogenation of fossil fuels requires the selection of a model fuel for use in two-phase experiments. While systems utilizing hexadecane have been used for similar studies in desulfurization, the low solubility of carbazole in hexadecane eliminates this solvent from serious consideration. 1-methylnaphalene is a good solvent for carbazole, is easily purchased, and forms an emulsion during testing that was easily broken by centrifugation. However, the observed toxicity of the solvent to many normally solvent-resistant strains of bacteria necessitated the search for a compatible biocatalyst for the model system. This initial screening indicates that at least one strain, *P. fraggi*, is capable of removing carbazole with 1-methylnapthalene as the model fuel. This strain may also prove more resistant to the toxicity of petroleum refining streams

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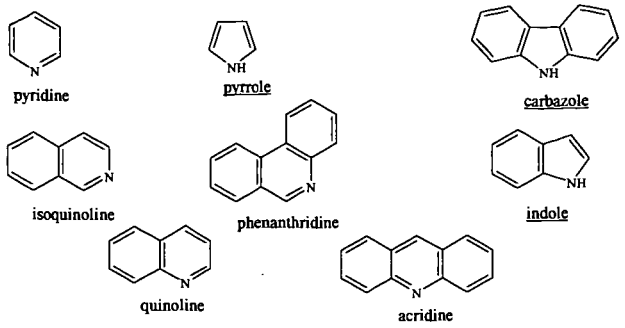

Figure 1: Examples of common nitrogen aromatic compounds found in fossil fuels. The nonbasic species are underlined.

Strain	Hexadecane system	1-methylnapthalene system
E. coli	+	-
P. sp. LD2	+	-
P. fraggi	+	+
P. mendicino	+	-
P. idaho	+	-
P. putida	+	-

Table 1. Tested strains harboring the carbazole degradation genes. Removal of carbazole from the model oil phase is denoted with a +.

BIOCATALYTIC UPGRADING OF PETROLEUM

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INTRODUCTION

Sulfur, nitrogen, metals and high viscosity in petroleum cause expensive processing problems in the refinery. Conventional technology does not exist to economically remove these contaminants from crude oil, so the problem is left for the refiners to handle downstream at a high cost.

Sulfur is the major concern for producers and refiners and has long been a key determinant of the value of crude oils for several reasons. First, sulfur presents a processing problem for refiners. Desulfurization offers refiners the opportunity to reduce the sulfur of their crude feedstocks before they ever enter the refinery system, minimizing downstream desulfurization costs. Secondly, the amount of sulfur in many finished products (i.e. diesel, gasoline) is limited by law. The regulations restricting allowable levels of sulfur in end products continues to become increasingly stringent. This creates an ever more challenging technical and economical situation for refiners as the sulfur levels in available crude oils continue to rise and creates a market disadvantage for producers of high-sulfur crudes. Lower-sulfur crudes continue to command a premium price in the market, while higher sulfur crude oils sell at a discount. Desulfurization would offer producers the opportunity to economically upgrade their resources.

Metals in petroleum lead to two major problems for the industry. Combustion of these fuels leads to the formation of ash with high concentrations of the metal oxides, leading to undesirable waste disposal issues. Also, when crude oil is refined, the metals are concentrated in the residual fraction. The residual fraction is often subjected to catalytic cracking where metals from the oil deposit on the cracking catalysts, resulting in the poisoning of the catalysts and decreasing their selectivity and activity. Like metals, nitrogen in oil also leads to the poisoning of the refinery catalysts and also results in increased nitrogen oxide emissions upon combustion in car engines.

High viscosity significantly hampers the pumping, transportation, refining and handling of petroleum. Common methods used to overcome problems associated with high viscosity include heating, dilution and chemical additives. All are expensive and require specialized equipment and/or safety procedures. Industry has long recognized the need for a safe, economical and effective method for reducing viscosity.

Biocatalytic processes for addressing these problems offers the petroleum industry potentially great rewards. Studies by Energy Biosystems Corporation (EBC) have focused on the removal of sulfur from crude oil and refinery streams by a microbial process, termed biocatalytic desulfurization (BDS). Furthermore, preliminary work has also been performed (and patented) on biocatalytic approaches to viscosity reduction and the removal of metals and nitrogen as additional approaches to fuel upgrading. Here, results of work performed on the biocatalytic desulfurization of crude oil will be presented.

MATERIAL AND METHODS

Materials and Equipment. 40 mL shake flasks and 500 mL batch stirred reactors (BSRs) were used to contact cells and crude oil. The reactor vessels are maintained at a constant temperature of 30°C by placing them in an incubator (shake flasks) or temperature controlled water jackets (BSRs).

Cells. Derivatives of the *Rhodococcus erythropolis* strain IGTS8 (ATCC 52986) used in these experiments are known for their ability to use organically-bound sulfur as the sole sulfur source. These cells catalyze the transformation of dibenzothiophene (DBT) to 2-hydroxybiphenyl (2HBP) and sulfate [1-3].

Oil. The crude oils used in the experiments were supplied by Texaco Exploration and Production Technology. Figure 1 shows chromatograms resulting from the sulfur chemiluminescence detector (SCD, sulfur-specific) of several crude oils. The sulfur contents range from 0.8 to 3.7 wt%. As a control, a model oil consisting of hexadecane (HD) and DBT dissolved to ~2600 ppm was utilized.

Procedures. A cell concentration of 12 to 50 g wet cell paste (WCP) per liter of total liquid volume contained in the reactor was utilized. Frozen cells were first added to sodium phosphate buffer (pH 7.5 at 0.156 mM) with 3% glucose. The cell slurry was placed in the reactor, agitated at 1000 rpm and sparged with 0.2 vvm air. The oil was added in the ratio of 1 part oil to 3 parts buffer. Shake flasks were taken down at specified times and BSRs were sampled at regular intervals to monitor sulfur concentrations. Samples were centrifuged at 39,000 x g for 10 minutes in order to separate the mixture of oil, water and cells. To ensure that any observed change was strictly cell-dependent, parallel experiments were performed without cells.

Analytical Methods. An HP 6890 gas chromatograph with electronic pressure control and detection by flame ionization detector (FID) and a Sievers model 350 flameless SCD was employed for crude oil analysis. The column was a Restek RTX-5, 15 meter, 0.25 mm ID with a 0.25 µm film thickness. The injection port was held at 340°C. The oven temperature program began at 50°C and was held for 2 minutes. The temperature was then increased by 15°C/minute to 320°C and was held for 10 minutes. A typical SCD chromatogram consists of a group of resolved peaks above a broad envelope of sulfur compounds (Figure 1).

A GC/MS SIM method for the quantitation of Cx-DBTs and Cx-benzonaphthothiophenes (Cx-BNTs) in crude oil was performed using a HP 5890 Series II plus gas chromatograph with electronic pressure control and mass spectrometric detection performed with an HP 5972 MSD. The column was a Restek RTX-1, 30 meter, 0.25 mm ID with a 0.5 µm film thickness. The injection port was held at 290°C. The oven temperature program began at 100°C, increased at a rate of 4°C/min to 315°C and held for 20 minutes.

Total sulfur quantitation of crude oils was performed with either a Horiba SLFA-1800H x-ray fluorescence analyzer or a Leco SC-444 Sulfur and Carbon Combustion Analyzer with infrared detection.

Sulfur XANES analyses were obtained at beam-line X-19A of the National Synchrotron Light source (NSLS), Brookhaven National Laboratory. Work was performed under contract with the University of Kentucky.

RESULTS AND DISCUSSION

Biocatalyst Development

The development of BDS for crude oil is complicated by the fact that the oil has a very wide boiling point range and that relatively little is known about the number and types of sulfur compounds and their concentrations present in crude oils. The state of EBC's analytical capabilities has greatly advanced to characterize crude oil including sulfur content, sulfur speciation and quantification, and physical properties. Many specialized techniques have been developed that have allowed us to gain valuable insight into the substrate specificity of the catalyst.

We have shown that the enzyme system in the *R. erythropolis* IGTS8 is extremely effective in transforming DBT, BNT, benzothiophene (BT) and their alkylated congeners in crude oil. Sulfur specific chromatograms of crude oil BDS samples reveal that the majority of these substrates have been removed (Figure 2). These results have been confirmed by GC/MS analysis developed to quantify the levels of the DBTs and BNTs in the crude oil (Figure 3). These methods have also revealed that the concentrations of these molecules were low in the crude oils tested and directly correlated to the amount of sulfur removed.

Attempts were made to characterize the sulfur species remaining after BDS (referred to as "Dsz recalcitrant material" or DRM). It was determined by XANES analysis that the majority of the sulfur in the DRM of this material is thiophenic (Figure 4) and, therefore, good targets for the IGTS8 catalyst.

A method to isolate and identify sulfur compounds from various oils and their DRMs was developed. The sulfur-containing species were selectively oxidized and converted to their corresponding sulfones. The sulfones were then separated from the hydrocarbon matrix by solid phase extraction (SPE). This powerful technique has allowed for the identification of the types of sulfur compounds remaining in the treated crude.

Other biocatalytic processes are under investigation that will result in crude upgrading. "Biocracking" has been investigated as a means to break down larger sulfur molecules so they will be small enough to enter the cell and to reduce the viscosity. Attempts were made to isolate organisms able to degrade compounds in the high molecular weight range. Proof-of-concept experiments were performed and validated with methylenebis-DBT (MBD). The goal was to isolate strains that could cleave the bond between the two DBTs. Soil samples were prepared and incubated with MBD. These enrichments were serially transferred, then plated to purify colonies. Individual isolates were obtained for further study.

In addition, if the sulfur bearing heterocycles contribute significantly to the viscosity of the oil, biocatalytic oxidative cleavage of at least one carbon-sulfur bond adjacent to the sulfur heteroatom(s) would result in the opening of the heterocyclic rings and sites of free rotation in the molecules formed effectively lowering the overall viscosity [4].

It has been shown that metals can be removed from crude by contacting the oil with an enzyme that degrades the metalloporphyrin molecules under conditions suitable for the removal and subsequent separation of the metals from the oil. The metals that were removed by the method [5] include nickel, vanadium, cobalt, copper, iron, magnesium and zinc.

Removal of nitrogen is also being investigated as an additional approach to fuel upgrading. This work is being performed at the University of Houston [6] and has been subsidized by EBC. Work has focused on the carbazole-degrading Pseudomonad LD2. Approaches in progress include isolation and characterization of the carbazole degradation enzymes, as well as the characterization and cloning of the genes encoding these enzymes.

The goal is to put all these catalytic activities together either in a single biocatalyst or a consortia to upgrade the petroleum by removing the sulfur, nitrogen, metals and reducing the viscosity in a single process step.

Process Development
Shake flask and BSR experiments were performed to address process concerns, such as reaction characteristics, separation characteristics, and catalyst stability and effectiveness. The effect of these parameters were determined in a series of assays designed to compare initial rates of desulfurization under a variety of process conditions. The assay results determined the optimum process conditions for BDS. The key parameters evaluated were water to oil ratio (WOR), catalyst to oil ratio (COR), mixing effects, oxygen demand, and temperature and pH optimum.

A process concept for the biodesulfurization of crude oil was developed based on the knowledge generated, a bench scale unit was constructed and proof-of-concept experiments were performed to develop a design basis specifying performance criteria, unit operations and process parameters for the biodesulfurization of crude oil. As part of the process flow diagram (PFD) development, a general description of the expected site conditions and product stream attributes was compiled. In addition, the process assumptions and equipment issues were delineated that were crucial to the design.

At this time, we envision a simple system capable of running with minimum operator intervention in the oilfield. The base case scenario for a field process is a batch reaction utilizing a pump and inductor for mixing and aeration. Separations will be performed with standard oilfield equipment, with desulfurized oil returning to storage and process water reinjected into a disposal well in the field. The spent catalyst will be inactivated and landfilled. The stored product oil would be tested for sulfur and other oil quality specifications prior to transport. Based on this process concept, the identified process parameters and assumptions, the attached PFD (Figure 5) was developed.

CONCLUSIONS

Significant progress has been made toward the commercialization of crude oil biodesulfurization. This progress includes the characterization of crude oil candidates for the BDS process; improved biocatalyst performance that directly relates to crude oil biodesulfurization; development of analytical methodology, which led to breakthroughs in the characterization of DRM; development of a process concept for crude oil BDS; and construction and testing of a prototype bench unit.

Technical hurdles still need to be overcome to achieve commercialization. The major obstacles to the economical biodesulfurization of crude oil include catalyst specificity and rate. Work continues to modify the catalyst to increase its effectiveness and to screen other organisms for additional desulfurization capabilities. In addition, mass transfer and separations hurdles must be overcome in crude oils with increased oil viscosity and density.

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Figure 1. Example SCD Chromatograms of Selected Crude Oils

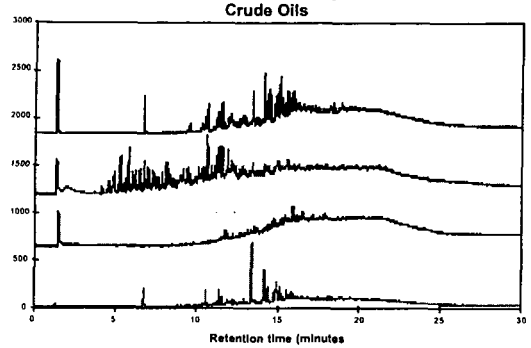


Figure 2. BDS of a Target Crude Oil

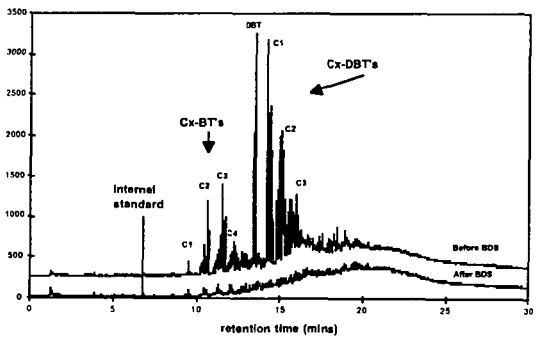


Figure 3. DBT Concentrations Before and After BDS for a Typical Crude Oil

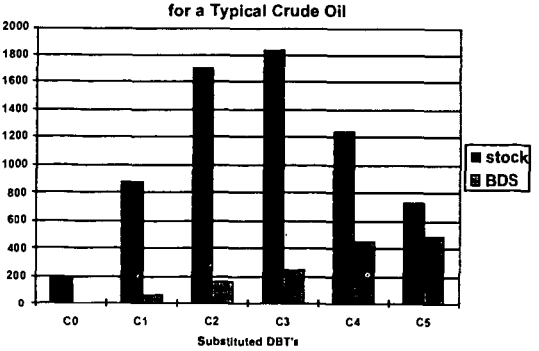


Figure 4. XANES Data for Various Petroleums

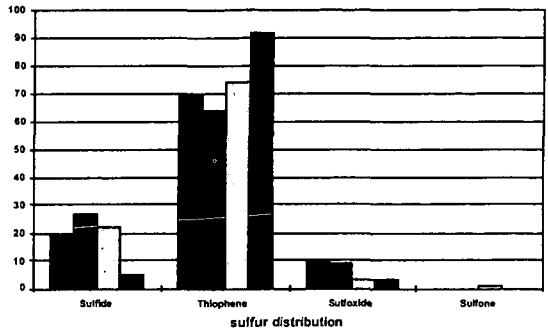
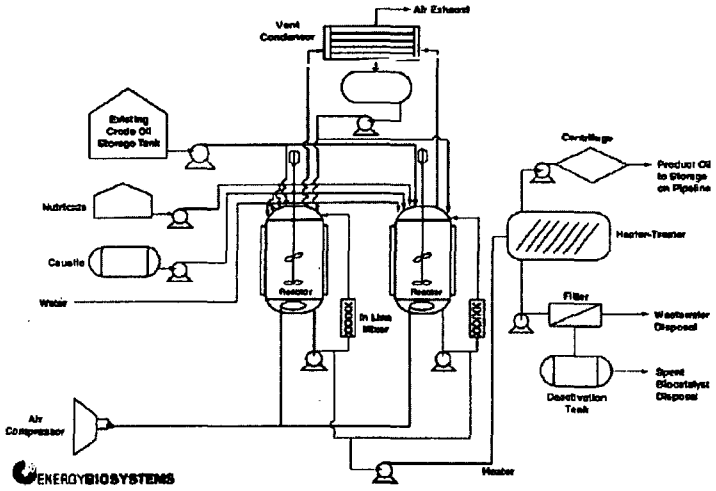


Figure 5: Proposed Process Flow Diagram for Crude Oil Biodesulfurization



PROCESS CONSIDERATIONS IN CRUDE OIL BIODESULFURIZATION

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ABSTRACT

Biodesulfurization offers an attractive alternative to conventional hydrodesulfurization due to the mild operating conditions and reaction specificity afforded by the biocatalyst. The enzymatic pathway existing in *Rhodococcus* has been demonstrated to oxidatively desulfurize the organic sulfur occurring in dibenzothiophene while leaving the hydrocarbon intact. In order for biodesulfurization to realize commercial success, a variety of process considerations must be addressed including reaction rate, emulsion formation and breakage, biocatalyst recovery, and both gas and liquid mass transport. This study compares batch stirred to electro-spray bioreactors in the biodesulfurization of both model organics and actual crudes using a *Rhodococcus* IGTS8 biocatalyst in terms of their operating costs, ability to make and break emulsions, ability to effect efficient reaction rates and enhance mass transport. Additionally, sulfur speciation in crude oil is assessed with respect to sulfur specificity of currently available biocatalysts.

KEY WORDS: crude oil desulfurization, *Rhodococcus*, electrostatic spraying, dibenzothiophene, biodesulfurization

INTRODUCTION

Biological processing of fossil fuel feedstocks offers an attractive alternative to conventional thermochemical treatment due to the mild operating conditions and greater reaction specificity afforded by the nature of biocatalysis. Efforts in microbial screening and development have identified microorganisms capable of petroleum desulfurization (see for example, [1-3]), denitrification [4], demetalization [4], cracking [5] and dewaxing. Biological desulfurization of petroleum may occur either oxidatively or reductively. In the oxidative approach, organic sulfur is converted to sulfate and may be removed in process water. This route is attractive due to the fact that it would not require further processing of the sulfur and may be amenable for use at the well head where process water may then be reinjected. In the reductive desulfurization scheme, organic sulfur is converted into hydrogen sulfide, which may then be catalytically converted into elemental sulfur, an approach of utility at the refinery. Regardless of the mode of biodesulfurization, key factors affecting the economic viability of such processes are biocatalyst activity and cost, differential in product selling price, sale or disposal of co-products or wastes from the treatment process, and the capital and operating costs of unit operations in the treatment scheme.

In all fossil fuel bioprocessing schemes, there is a need to contact a biocatalyst containing aqueous phase with an immiscible or partially miscible organic substrate. Factors such as liquid / liquid and gas / liquid mass transport, amenability for continuous operation and high throughput, capital and operating costs, as well as ability for biocatalyst recovery and emulsion breaking are significant issues in the selection of a reactor for aqueous / organic contacting. Traditionally, impeller-based stirred reactors are utilized for such mixing due to their ease of operation and wide acceptance in the chemical and biological processing industries. Such mechanically stirred reactors contact the aqueous and organic phases by imparting energy to the entire bulk solution, i.e. the impeller must move the contents of the reactor.

Recent advances in the area of contactors for solvent extraction have lead to the development of electrically driven emulsion phase contactors (EPC™) for efficient contact of immiscible phases [6]. In this concept, the differing electrical conductivity between the aqueous and organic phases causes electrical forces to be focused at the liquid / liquid interface, creating tremendous shear force. This shear causes the conductive phase to be dispersed (5 □m droplet size) into the non-conductive phase, but does so with decreased energy requirements relative to mechanical agitators due to the fact that energy is imparted only at the liquid / liquid interface and not the entire bulk solution. In a configuration of the EPC™ developed at the Oak Ridge National Laboratory, the contactor serves to disperse aqueous phase containing biocatalyst into an organic

phase. The EPC™ creates droplets of water containing biocatalyst ~5 □m in diameter within an organic phase.

Here, we compare the performance of the EPC™ to that of a batch stirred reactor (BSR), investigate the required level of biocatalyst activity before the surface area afforded by the EPC™ becomes a factor in reactor performance, and characterize the emulsion formed by both reactors in the presence of bacteria. We have investigated the emulsion quality formed in the EPC, evaluated the power requirements and analyzed the mass transfer issues in comparison to stirred reactors. Results on biodesulfurization of actual crude oil by wild type *Rhodococcus* IGTS8 are also included. Finally, we assess the sulfur specificity of available biocatalysts with respect to sulfur compounds present in crude oils.

MATERIALS AND METHODS

The experimental procedures used for studying biodesulfurization in model systems have been discussed in detail in previous publications [7-9]. A detailed description of oil experiments is provided here.

Biodesulfurization of Van Texas Crude oil

Biodesulfurization of Van Texas crude oil was studied in batch stirred reactors to evaluate the substrate specificity of the biocatalyst. The experiment was conducted over a treatment period of 6 days. The crude had an API specific gravity of 31°, and a sulfur content of 0.96 wt.%. The crude oil did not contain volatiles due to production at elevated temperature (~99°C). Experiments were performed in batch stirred reactors utilizing 50 g of frozen *Rhodococcus sp.* wild type strain IGTS8 (ATCC 53968) cell paste which were brought up to 750 mL with 0.156M (pH 7.5) phosphate buffer. The cells were suspended in the phosphate buffer prior to addition to the reactor. The reactor vessel used was a 1-L VirTis Omni-Culture fermentor (model 178657, Gardiner, NY), utilizing a 6-bladed Rushton-type impeller with 2 baffles. The reactor was kept at 30^{o}C, agitated at 800 RPM, and aerated with room air at a rate of 0.2 standard liters per minute (SLPM). A water condenser was used on each reactor to capture volatiles which were expected to be minimal or non-existent considering the fact that the operating temperature was much less than that of the oil reservoir. The experiment was conducted with 250 mL of crude oil, treated with 750 mL of the aqueous phase. Samples (30 mL from the top of the organic phase) taken during the course of biological treatment were collected after ceasing the agitation and aeration for 5 min to allow the aqueous and organic phases to separate. The reactor contents were emptied at the end of the run and centrifuged at 6000 rpm in a Beckman Model TJ-6 centrifuge to obtain a sample of treated crude oil. Closed samples were boiled in a closed container for 30 min to halt biological activity.

Analytical

Model system experiments

In the experiments reported here, DBT and 2-HBP concentrations in the aqueous phase were below our levels of detection. DBT and 2-hydroxybiphenyl (2-HBP) concentrations in n-hexadecane were measured by gas chromatography using a Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector.

Crude oil

A GC-SCD method was used to determine the sulfur content of the aromatic fraction of the oil. To allow facilitated observation of sulfur in the treated oil, whole oil samples were fractionated according to ASTM method D2007. An extended ASTM D2887 procedure was used for chromatographic separation of the aromatic fraction of the crude oil. Sulfur analysis was performed by modifying the ASTM D2887 procedure by adding a Sievers Chemiluminesence sulfur specific detector after the flame ionization detector.

RESULTS AND DISCUSSION

Rate of biodesulfurization

The specific rate of DBT desulfurization by *Rhodococcus sp.* was typically between 1 and 5 mg 2-HBP produced per dry g of biocatalyst per hour. Specific rates of 2-HBP production in the batch stirred reactor and the EPC™ reactor systems were within experimental variance and no

appreciable difference in desulfurization rates were seen between the two reactors. Due to the high surface area reported in the EPC™ [7], higher rates were expected in the EPC™, however, similar performance was observed in both reactors. The reaction rate obtained was without any supplemental carbon or energy source. Note that the only available carbon and energy source for the biocatalyst other than what may be carried over in the frozen cell paste, was hexadecane and DBT. However, DBT was not used as the carbon source by the biocatalyst, since the end product of DBT conversion was 2-HBP (thus preserving the carbon number and fuel value). Other studies (outlined in [7]) have utilized additional external carbon and energy sources and have reported higher activities with *Rhodococcus sp.* A commercial scale biodesulfurization process may require a higher cell density to achieve maximum conversion in a minimum time, provided it does not affect yield with respect to biocatalyst usage. In order to study effect of cell density, experiments were conducted in the BSR at different cell loadings. This experiment also lead to determination of the cell density at which point the BSR becomes mass transfer limited. A following experiment in the EPC™ at this cell density was conducted to evaluate the benefits of high surface area afforded by the EPC™.

Mass transport issues

The rate of desulfurization, when normalized with respect to cell mass, was found to decrease with increasing cell density indicating that mass transfer resistance was the controlling process in desulfurization. A statistical analysis of the data indicated mass transfer limitation between 5X and 10X cell density in the BSR. The mass transfer limitation may be due to gas-liquid or liquid-liquid mass transport resistance.

The results of experiments conducted in the BSR at 10X cell density indicated no gas-liquid mass transfer limitation. Increasing the rate of air supply or increasing the oxygen tension in the reactor through the use of pure oxygen rather than air did not seem to affect HBP production. This suggests that the system may be limited by liquid-liquid mass transfer. Since the EPC™ reportedly provides larger liquid-liquid interfacial area, the BSR was compared with the EPC™ for desulfurization activity at the high cell density.

Comparison of the EPC™ and BSR performance at 10X cell density showed no difference in the desulfurization rates between the two reactors. Thus, either the system is not truly mass transport limited or the EPC™ did not provide a larger surface area for reaction under the present conditions. A detailed characterization of the emulsions formed in the BSR and EPC™ in the presence and absence of biocatalyst was conducted and is reported below.

Emulsion quality in BSR and EPC™

A detailed drop size analysis of the two-phase emulsion formed in the BSR has been reported previously [7]. Characterization of the emulsion quality in BSR in the absence of biocatalyst has revealed 100-200 micron droplets under the conditions of experiments conducted here. The droplets formed in the EPC; however, are in the 1-10 micron range. The ability to form fine emulsions in the EPC™ without increasing energy utilization (see energy utilization section below) could have tremendous impact upon processing costs assuming that the biocatalyst utilized is active enough to be mass transport limited.

Emulsion quality in the presence of biocatalyst

Due to the opaqueness rendered by presence of biocatalyst, observations could not be made *in situ* during reactor operation. To determine the emulsion quality formed in the EPC™ and BSR, and to determine whether the EPC™ offers larger surface area than BSR, samples were collected from the reactors and observed under a microscope using a 100x oil emersion objective. Microscopic examination of samples showed formation of a very fine emulsion in both reactors with droplet sizes ranging from 1 to 10 □m. Formation of such an emulsion in the BSR may be presumed due to production of biosurfactants by the biocatalyst IGTS8. Average droplet size for EPC™ and BSR samples were 2.54 ± 2.40 □m and 3.08 ± 1.78 □m, respectively (n>300). Further, a significant amount of the biocatalyst was extracted and existed in the organic phase. Thus, a very fine emulsion is formed in the EPC™ as well as the BSR, and it appears that it is for this reason that an augmentation in desulfurization rate is not seen in the EPC™ relative to the BSR. A couple of process issues warrant consideration here. Firstly, due to the formation of a fine stable emulsion, downstream separation of the multiphase mixture to obtain clean organic fuel may require additional separation processes. Secondly, the *Rhodococcus* biocatalyst used in these experiments was extracted into the organic phase. If biocatalyst recovery and reuse is desired, separation of the biocatalyst from aqueous as well as organic phases will be required.

Energy utilization by EPC™

Typically, stirred reactors or impeller based reactors are capable of achieving water or oil droplet sizes of 100 -300 □m in diameter under the conditions used in this study when surfactants are not present. Tin order to create such droplet size distribution, the energy required is on the order of 1-6 W/L (based upon empirical correlation's [10]). It is estimated that if impeller based systems were capable of producing 5 □m droplets, it would require ~25 kW/L [11] if surfactants are not present. The EPC™ creates droplets of water containing biocatalyst ~5 □m in diameter within an organic phase, and does so with a power requirement of 3 W/L [7]. Thus, if a high activity biocatalyst is available, which is actually limited by mass transport, the EPC™ could result in tremendous savings over the batch stirred reactor. For instance, on a 1 L basis, a BSR using a 3:1 water to oil ratio and producing oil droplets of 150 mm in diameter creates 1×10^5 cm^2 of interfacial surface area. On the same volume basis, an EPC™ creating 5 □m diameter aqueous droplets and having a 5% aqueous hold-up creates 6×10^5 of interfacial area at $1/15^{th}$ the aqueous volume to do so. In a mass transport system, the rate of desulfurization would thus be expected to be six times as large using 93% less biocatalyst. An additional important point which needs to be noted here is that the fine emulsion formed in the EPC™ is an unstable emulsion i.e., the emulsion breaks easily upon removal of the electric fields giving easy separation of the organic and aqueous streams.

Crude oil biodesulfurization

Oil samples collected from the BSR were analyzed by GC-SCD to obtain the distribution of organosulfur compounds in the crude oil. Analysis was done on the aromatic fraction of the oil and not the whole oil, since the baseline did not return to its initial value in case of the whole oil. This fraction of the Van Texas oil accounted for 22% of the oil's original volume. As shown in Figure 1, the total sulfur content of this aromatic fraction was reduced from 3.8 to 3.2% in 6 days of treatment with IGTS8.

The results indicate removal of comparatively low molecular aromatic sulfur compounds; however, a large portion of the organosulfur fraction does not seem to be affected by the biodesulfurization process. Additional analysis by GC-MS (not shown here) has revealed up to 90% sulfur removal from DBT and substituted DBT compounds. While it appears that this biocatalyst is capable of desulfurizing the majority of sulfur species present in diesel (DBT and substituted DBT compounds) and that only improvements in the rate of desulfurization are needed for the commercialization of this process, a great deal of research is needed for oil biodesulfurization to be realized. The sulfur specific oxidation of DBT by *Rhodococcus* resulted from over 15 years of research using DBT as the model organic sulfur compound in coal and oil. Detailed sulfur speciation studies and biocatalyst development is needed to achieve desulfurization of the broad spectrum of organic sulfur species present in crude oil and to realize the promises of petroleum biodesulfurization.

CONCLUSIONS

A variety of process considerations in the biodesulfurization of petroleum feedstocks were addressed in this study including reaction rate, emulsion formation and breakage, biocatalyst recovery, and both gas and liquid mass transport. Comparison of batch stirred reactor to EPC™ revealed formation of high surface area in the EPC™ in the absence of surface-active agents. Presence of biocatalysts capable of producing biosurfactants results in fine emulsions in both reactors; however, poses a potentially more difficult problem with downstream multiphase separation. The use of EPC™ as a biodesulfurization reactor can result in up to several orders of magnitude energy savings over BSR in the absence of surfactants. Gas-liquid mass transfer was not a limiting factor in biodesulfurization studies with model systems. Further, biodesulfurization experiments with actual crude oil showed that presently available biocatalysts such as *Rhodococcus* sp. IGTS8 are capable of removing DBT and substituted DBT type compounds but do not affect the remaining portion of the organosulfur compounds. Thus, there is a need for further development in biocatalysts capable of desulfurization of higher molecular weight non-DBT type sulfur compounds present in crude oil.

ACKNOWLEDGMENTS

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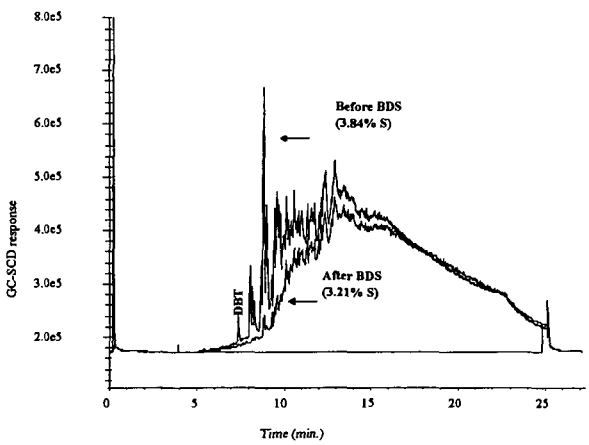

Figure 1. Analysis of the aromatic fraction of Van Texas crude oil by GC-SCD. The biotreatment results in removal of low molecular weight DBT-type compounds; however, the higher molecular weight compounds are not affected.

BACTERIAL DESULFURIZATION STUDIES OF ORGANOSULFUR-ENRICHED MAYAN CRUDE OIL EXTRACTS USING LIQUID CHROMATOGRAPHY/ATMOSPHERIC PRESSURE CHEMICAL IONIZATION/ MASS SPECTROMETRY

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INTRODUCTION

The sulfur content in coal and petroleum products ranges from 0.025 to 11%, thus creating a potential problem through the production of sulfur dioxide which is a major component in acid rain.[1] As fossil fuel consumption increases, governments are forced to implement stricter controls on sulfur dioxide emissions in order to reduce the occurrence of acid rain. A regulatory burden will necessitate the development of methods which can be used to lower the sulfur content of fuels, both before and after processing. Inorganic sulfur can be successfully removed by a variety of physical separation methods. However, sulfur removal from organosulfur compounds is more difficult to achieve. The current methodology, hydrodesulfurization, involves the use of inorganic catalysts at high temperature and pressure to generate desulfurized hydrocarbon. The approach is expensive, produces hydrogen sulfide, and is ineffective for many classes of organosulfur compounds. Another approach , which has been well established by the work of Kilbane, involves bacterial systems, including *Rhodococcus* IGTS8, for the bacterial desulfurization of coal and petroleum[2-7]. These systems offer many advantages over hydrodesulfurization including the use of much milder conditions, and the generation of water-soluble sulfite or sulfate products as well as desulfurized hydrocarbon. Most importantly, bacteria are able to remove sulfur from a much broader range of organosulfur compounds.

In order to assess the efficacy of bacteria for the desulfurization of crude oil, we have developed a protocol which involves the isolation of organosulfur compounds from a Mayan crude oil, followed by the analysis of the extract using liquid chromatography/ atmospheric pressure chemical ionization/ mass spectrometry (LC/APCI/MS). Concurrently, the organosulfur isolate is used as a food source for the bacterium, *Rhodococcus* sp. IGTS8. By analyzing the mass spectrum before and after bacterial inoculation, we hope to delineate the specificity of the bacteria toward the range of organosulfur compounds that are found in petroleum products.

EXPERIMENTAL

The Maya crude oil (density = 0.91 g/mL) was provided by Mobil Oil Corporation from their Beaumont, TX refinery. The Maya crude was characterized by a series of distillations and was found to contain: 38% light distillate (the fraction that distills below 200^{0}C under 1 atm pressure), 22% middle distillate (the remaining fraction that distills below 180^{0}C under 20 torr external pressure) and 22% residue (the fraction that remains). The corresponding densities were 0.804 g/mL for the light distillate, 0.917 g/mL for the middle distillate and 1.013 g/mL for the residue.

A standard mixture of polyaromatic hydrocarbons (PAH: 7.51 mM flourene, 6.78 mM fluoranthene, 6.57 mM phenanthrene, 4.94 mM pyrene, 5.39 mM chrysene, 4.28 mM benzo[k] fluoranthene) and 10. 37 mM dibenzothiophene (DBT) was prepared in dichloromethane.The mix of PAH and DBT would be used in the evaluation of the ligand exchange protocol.

Alumina (Brockman Activity I, 80-200 mesh, Fisher, Fairlawn, N.J.) was dried at 200^{0}C overnight. CuCl/silica was prepared by mixing approximately 100 g of silica gel (100-200 mesh, Fisher) with 5 g of cupric chloride (Aldrich) in distilled water, drying the mixture to a wet powder with a rotary evaporator, and then drying at 200^{0}C in an oven for 24 h prior to use.[9] PdCl/silica was prepared by mixing approximately 100 g of silica gel (100-200 mesh, Fisher) with 5 g of palladium (II) chloride (Aldrich) suspended in an aqueous solution, drying in an oven at 95^{0}C overnight, then holding at 200^{0}C for more than 24 h prior to use. [10]
Approximately 0.2-0.5 g of the Maya crude oil was dissolved in 5 mL of methylene chloride then adsorbed onto 3 g of neutral alumina. The solvent was removed from the alumina by vigorously stirring the mixture under a gentle stream of dry nitrogen gas. The alumina with the adsorbed sample was then packed on top of 6 g of neutral alumina in an 11 x 300 mm. The sample was eluted with the following chromatographic grade solvents: 20 mL of hexane which removes aliphatic hydrocarbons (designated as fraction A-1) ; 50 mL of benzene which removes poly aromatic hydrocarbons (designated as fraction A-2) Figure 1. illustrates the entire fractionation and isolation scheme for the sulfur compounds.[11]

Fraction A-1 was adsorbed onto 0.5 g of the CuCl /silica gel then packed on top of 5 g of CuCl /silica gel in an 11 x 300 mm column. 50 mL of n-hexane were added in order to elute the aliphatic hydrocarbons (designated as fraction C-1) [9]. One hundred mL of chloroform/diethyl ether (9:1 , v: v) were then used to elute the aliphatic sulfur hydrocarbons (designated as fraction C-2). See Figure 1.[9]

Fraction A-2 was adsorbed onto 0.5 g of the PdCl/silica gel then packed on top of 5 g PdCl/silica gel in an 11 x 300 mm column. Thirty mL of chloroform/n-hexane (1:1) were used to elute the polyaromatic hydrocarbons (PAH, designated as fraction P-1). A further fifty mL of the same eluent , chloroform/n-hexane (1:1), were used to elute the polyaromatic sulfur heterocycles (PASH, designated as fraction P-2). One hundred mL of chloroform/diethyl ether (9:1) were used to elute other sulfur polyaromatic compounds (S-PAC, designated as fraction P-3). Fractions P-2 and P-3 were reduced in volume to approximately 1 mL by rotary evaporation, after which 50 µL of diethylamine were added ,in order to break up the Pd complexes. Fraction P-3 was further cleaned by passing it through neutral alumina with 50 mL of benzene.[12] Fractions P-1, P-2, and P-3 were evaporated to dryness and redissolved in 5 mL of methylene chloride. See Figure 1.

The PAH and DBT mixture was analyzed using a Beckman 110 B solvent delivery system, an Altex 210A injection valve, and a Beckman Model 160 absorbance detector set at 254 nm. The HPLC system attached to the Finnegan LCQ included a Model P4000 pump and a Model AS3000 autosampler (Thermo Separation Products Inc., San Jose, CA.). The analytical column employed for both HPLC systems was a LiChrosorb Amino (4.6 x 150 mm, dp = 5 um) column (Phase Separations, Franklin, MA). The mobile phase employed for the separation of the standard mixture was 100% hexane while the mobile phase employed for the LC/APCI/MS analysis consisted of n-hexane and methylene chloride on a gradient from 100% n-hexane to (60:40) n-hexane/methylene chloride over a period of 6 minutes. For both HPLC systems, the flow rate was constant at 1.0 mL/minute, the column temperature was ambient, and the sample volume was 20 uL.

Atmospheric pressure chemical ionization (APCI/MS)was performed on an LCQ ion-trap mass spectrometer equipped with an APCI source (Finnigan MAT, San Jose, CA). The APCI source parameters were as follows: discharge voltage, 5 kV; vaporizer temperature, 450 C; nitrogen sheath gas, 80 psi; nitrogen auxiliary gas,10 psi; heated capillary, 150 C; capillary voltage, 5 V; and tube lens voltage, 5 V. NAVIGATOR Version 1.2 software (Finnigan MAT, San Jose, CA). was used for sample acquisition and data reduction.

The bacterial strain *Rhodococcus erythropolis* sp. IGTS8 was obtained from ATCC (ATCC 53968). Two different media were used for the growth of *Rhodococcus* IGTS8: a Difco nutrient media and a sulfur-free minimal media designated BSM2.[8] *Rhodococcus* was grown to saturation in overnight tubes containing nutrient media. The cells were then concentrated by centrifugation and washed twice with BSM2 media, before suspension in 100 mL of BSM2 media. The sulfur source necessary for growth was excluded in the negative control, was 200 µM dibenzothiophene (DBT) in the positive control, or was an aliquot of the sulfur-containing extract from crude oil. Growth was monitored by optical density measurements at 600 nm using a Beckman Model 7500 ultraviolet-visible spectrophotometer.

RESULTS AND DISCUSSION

The standard mixture of the PAH and DBT was fractionated according to the ligand exchange chromatography scheme illustrated in Figure 1. Fraction P-1 exhibited the PAH compounds as well as some of the DBT as previously noted.[13]

Fractions P-2 and P-3 did not exhibit any PAH compounds, thus validating the efficacy of the protocol for the isolation of sulfur compounds.

Both the Maya crude oil and the residue were also fractionated using ligand exchange chromatography. Fractions P-2 and P-3 from the residue were then added to *Rhodococcus erythropolis* sp. IGTS8. No bacterial growth was observed. Most of the polyaromatic sulfur heterocycles (PASH) and other sulfur polyaromatic (S-PAC) compounds are in the light and middle distillates and so this result was expected. The implication however is that *Rhodococcus erythropolis* sp. IGTS8 may not be very effective in the biodesulfurization of Maya crude oil residue.

Fractions P-2 and P-3 from the crude oil were investigated using LC/APCI/MS. Each fraction gave a single broad peak under the chromatographic conditions employed. Any attempt to use a

weaker mobile phase (e.g., 100% hexane) in order to better resolve the multi-component mixture results in the precipitation of some of the components from the mixture. A mass/charge (m/z) scan of the chromatographic peak for each fraction results in a broad mass spectral envelope. Fraction P-2 shows significant intensity extending from about 400 to 1600 m/z (the mass spectrum (MS) is shown in Figure 2). The first statistical moment of the distribution lies around 1000 m/z with local maxima at about 850, 1100 and 1220 m/z. The distribution can be regarded as a fingerprint for the polyaromatic sulfur heterocycles (PASH) in the Maya crude. The mass spectrum (MS) of fraction P-3 shows significant intensity extending from about 400 to 2000 m/z. The distribution features a maximum around 900 m/z. This distribution can be regarded as a fingerprint for the other sulfur polyaromatic compounds (S-PAC) in the Maya crude. Both m/z scans contain multiple peaks, possible fragments, and possible long-lived adducts from the nebulization and chemical ionization process; however, with further method developments in the fractionation of sulfur compounds, the selection of other sorbents and mobile phase eluents for optimal liquid chromatographic resolution, coupled with mass spectral optimization of the nebulization and ionization process, as well as the implementation of MS^n acquisitions, we hope to further delineate the distribution and composition of the P-2 and P-3 fractions.

Fractions P-2 and P-3 from the crude oil will also be added to the *Rhodococcus erythropolis* sp. IGTS8. Bacterial growth will be monitored for up to 30 days. An internal standard will be added to the aliquots and the loss of intensity at individual m/z values will be monitored. Those signals which exhibit a significant loss of intensity will be further explored via MS^n experiments in order to assess the chemical structure.

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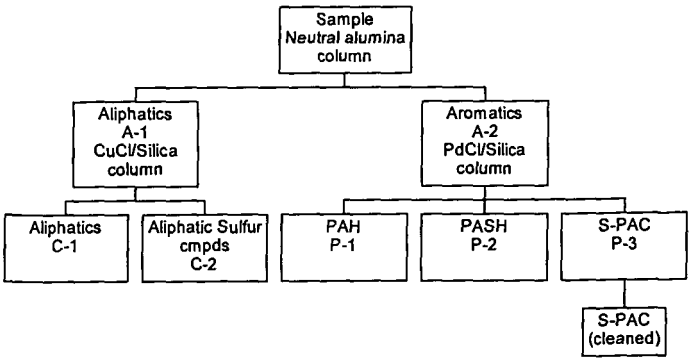

Figure 1. Fractionation and Isolation Scheme for Sulfur Compounds

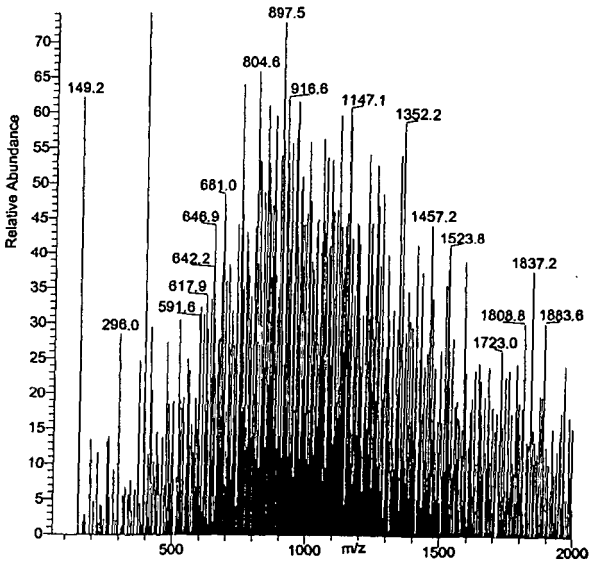

Figure 2. Mass Spectrum of the P-2 Crude Oil Fraction.

BIODESULFURIZATION GENE EXPRESSION BY PROMOTER REPLACEMENT IN *RHODOCOCCUS*

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INTRODUCTION

Biodesulfurization is a pathway-driven and enzymatic process, best known in select strains of *Rhodococcus*, that removes sulfur specifically from sulfur heterocycles such as dibenzothiophene (DBT) present in fossil fuels (*1-6*). The ability of several rhodococcal strains to desulfurize is thus far a plasmid-encoded phenotype involving three genes (*soxABC* [also known as *dszABC*]) and at least a chromosomal gene product which provides the reduced flavin and oxidized NADH required for the two initial oxygenase activities (Fig. 1). The end products of this sulfur-specific removal scheme are liberation of inorganic sulfur as sulfate or sulfite and production of 2-hydroxybiphenyl (2-HBP) without disruption of the original aromatic rings. The practical advantages of this biodesulfurization process include unnecessary loss of calorific values of the fuel upon combustion and a reduced emission of harmful sulfur dioxide into the atmosphere (*1, 4, 5, 11-13*).

We are interested in developing an efficient desulfurization biocatalyst based on a native *Rhodococcus* strain. This strategy, while not compromising the possible advantages offered by the *Rhodococcus* background, necessitates a resolution of the repression problem faced by the sulfur oxidation genes when the native cells are grown in the presence of sulfur-containing compounds (*1, 3, 7, 14, 15*). Here we report a promoter replacement strategy and the results of a preliminary study showing the feasibility of *sox* gene expression in a rich and sulfate-proficient background.

EXPERIMENTAL AND DISCUSSION

Bacterial strains and shuttle expression vector

The rhodococcal host used in this study is a plasmid-free derivative of the desulfurizing strain *Rhodococcus* sp. X309 (ATCC 55309), designated *Rhodococcus* sp. strain X309-10-2 (henceforth strain 10-2; ref. *3*). The *sox* phenotype was scored by growth on DBT as a sole sulfur source and the fluorescence formed around patches of bacteria on a DBT plate spray assay (*3, 7*). Bacterial growth media including minimal salt medium (MSM) and general recombinant DNA techniques were as previously described (*3, 15, 16*).

Plasmid pKSΔ6-1 (Fig. 2) is capable of replication in both *Escherichia coli* and *Rhodococcus* by virtue of the origin of replication from the *E. coli* pBluescript II KS- vector and the replication region of the pSOX desulfurization plasmid from strain X309 (*16*). For selection the gene markers are ampicillin (Ap; 50 μg/ml) resistance and chloramphenicol (Cm; 30 μg/ml) resistance, respectively. The pBluescript KS vector component also provides the blue-white color selection when the cells are plated on indicator plates containing IPTG and X-gal. Previously, we found that the levansucrase-encoding gene (*sacB*) from *Bacillus subtilus* (*17*) could be expressed in *Rhodococcus* and served as a counterselectable marker in the shuttle expression plasmid designated pKSΔ6-1*sac* (*16*). Importantly, the *sacB* promoter is not known to be repressed by sulfate, a feature that is explored in the following construct.

Promoter replacement for expression of soxABC genes in *Rhodococcus*

The concept is to replace the sulfate-repressed *sox* promoter (*14*) by that of *sacB* to drive the expression of *soxABC* genes. Knowledge of the precise sequence of these DNAs was used to design primers for PCR (polymerase chain reaction) amplification and cloning of the specific DNA fragments in the core shuttle plasmid pKSΔ6-1 by a step-wise procedure. i). The *sacB* promoter fragment was amplified by the following primers using pUM24 plasmid as template (*18*). Primers SacBpro5' (5'-CGCAGGGCCCATCACATATACCTGC) and SacBpro3' (5'-

GCTGACTAGTCATCGTTCATGTCTCCTT) were designed to contain the *Apa*I and *Spe*I restriction sites (boldfaced), respectively. The underlined bases correspond to nucleotides 13-30 and 467-448 of the *sacB* noncoding sequence, respectively (*17*). Both primers have a calculated melting temperature of 54 ^{0}C. The CAT sequence adjacent to the *Spe*I site specifies the complementary sequence of the initiator codon of *sacB*. ii). The 3.7-kb DNA fragment of strain X309 pSOX plasmid containing the *soxABC* genes was amplified based on the sequence derived from the *Rhodococcus* sp. IGTS8 strain (*7*). The forward oligomer contains the sequence 5'-CCTGACTAGTCAACAACGACAAATGCATCT (*Spe*I site underlined), and the sequence of the reverse oligomer is 5'-GTCTCTAGATCAGGAGGTGAAGCCGGG (*Xba*I site underlined). The sequence 3' to the *Spe*I site represents the third to eight codons of the *soxA* sequence. In the reverse oligomer the TCA triplet represents the complement of the *soxC* stop codon.

Digestion of the purified *sacB* promoter-containing fragment by *Apa*I and *Spe*I endonucleases allowed specific and directional cloning at the MCS of the pKSΔ6-1 vector (Fig. 2). After plasmid isolation the *soxABC*-containing fragment was cloned at the *Spe*I and *Xba*I sites of the MCS after generation of the compatible restriction ends (Fig. 2).

As a result of transformation in *E. coli* strain DH10B, recombinant plasmids were isolated and checked for authenticity of the clones. A straightforward double digestion by *Spe*I and *Xba*I would verify the correct size of the insert. But it was necessary to first transform the plasmid in an *E. coli dam*-minus strain since in our primer design we had inadvertantly introduced an adenine methylation sequence adjacent to the *Xba*I cleavage site (TCTAGATCA; methylated base underlined). The orientation of the clone was verified by *Not*I restriction, an unique site within the *soxB* gene (Fig. 2). Using SacBpro5'as primer the DNA sequence encompassing the cloned junction was also verified.

Expression of soxABC genes in *Rhodococcus* and metabolite analysis

Two independent clones of *soxABC* genes (clone #4B and #7) of the above constructs were transformed into rhodococcal strain 10-2 by electroporation using the conditions and Bio-Rad Gene Pulser apparatus as previously described (*16*). The recombinants were selected on MSM media plates containing DBT (0.52 mM; Aldrich Chemical Co., Wilwaukee, WI) and Cm (30 μg/ml). The *sox* phenotype was first examined under UV for fluorescence around patches of bacteria on a DBT spray plate assay. Neither the plate nor the liquid culture imparted fluorescence. This could be due to low level of expression or no expression at all. To check for these possibilities, metabolites of DBT transformation were analyzed by the solid phase microextraction (SPME) technique coupled to GC-MS (*19*). The solventless SPME technique is known for its speed and sensitivity, detection in the microgram per liter range. Sample preparation and analysis of metabolites were carried out as previously described (19). As a result, although the peaks are minor (not shown), both 2-HPB (m/z 170) and DBT-sulfone (m/z 216) were detected as metabolites besides the starting material DBT (m/z 184). Extracts of cells grown in the rich Luria Bertani (LB) broth were also analyzed. Similar pattern was observed for the recombinant strain but not the native X309 strain which is known to be repressed by the presence of sulfates. As a positive control, the plasmid-free 10-2 mutant strain did not yield any of the characteristic products.

CONCLUSIONS

This preliminary study showed the feasibility of expression of sulfur oxidation genes in *Rhodococcus* by a heterologous promoter that is not repressed by the presence of sulfate. Undoubtedly, the shuttle expression system provides a good base for further improvement and optimization. Recently, recombinant *Pseudomonas* strains have been constructed to carry out an improved level of biodesulfurization (*20*).

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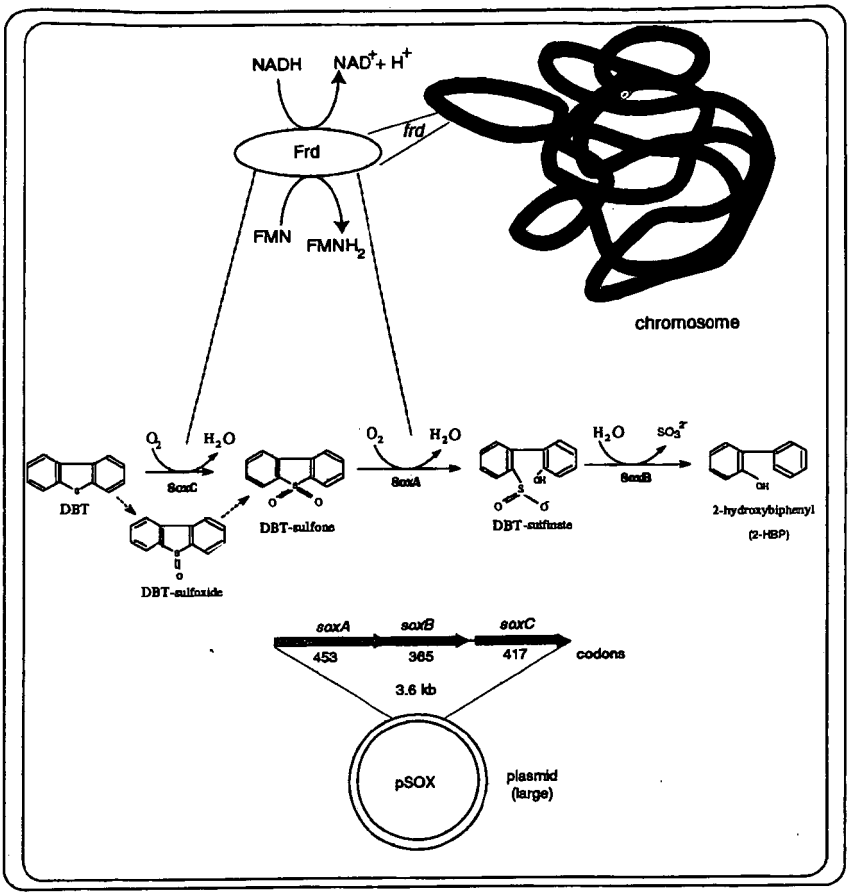

Fig. 1 The sulfur oxidation (*sox*)/desulfurization pathway of dibenzothiophene (DBT) and its genes in *Rhodococcus* spp. SoxA, DBT sulfone monooxygenase; SoxB, 2-(2-hydroxybiphenyl)-benzenesulfinate; SoxC, sulfite/sulfoxide monooxygenase; Frd, flavin reductase (FMN-NADH oxidoreductase). Compiled from references *6-11*.

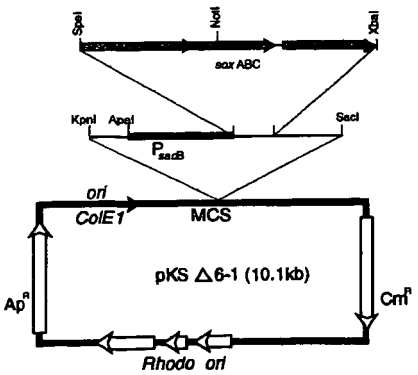

Fig. 2 Step-wise construction of the *sox*-gene expression plasmid under the control of the *Bacillus subtilis sac*B promotor element (P*sac*B). MCS is the multiple-cloning site of the *Rhodococcus-Escherichia coli* shuttle plasmid pKSΔ6-1 as previously described (16). Cm^R, chloramphenicol resistance gene; Ap^R, ampicillin resistance gene. *Rhodo ori* and *ColE1 ori*, origins of replication of *Rhodococcus* sp. strain X309 and *E. coli*, respectively.